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**Structural study of *Carcinus maenas* hemocyanin by native ESI-MS:**

**interaction with L-lactate and divalent cations**

Short title: Study of *C. maenas* hemocyanin by ESI-MS

Keywords: dissociation, macromolecule, mass spectrometry, non-covalent interactions, physiological modulator, reassociation

Matthieu Bruneaux<sup>1,2</sup>, Peran Terrier<sup>3,4</sup>, Emmanuelle Leize<sup>3</sup>, Jean Mary<sup>1,2</sup>, François H. Lallier<sup>1,2</sup>, Franck Zal<sup>1,2\*</sup>

<sup>1</sup>UPMC Univ. Paris 06, UMR 7144, Equipe Ecophysiologie : Adaptation et Evolution Moléculaires, Station Biologique de Roscoff, 29682 Roscoff, France

<sup>2</sup>CNRS, UMR 7144, Station Biologique de Roscoff, 29682 Roscoff, France

<sup>3</sup>CNRS-ULP, UMR 7177, Laboratoire de Dynamique et Structure Moléculaire par Spectrométrie de Masse, Institut de Chimie, ISIS, 67083 Strasbourg, France

<sup>4</sup>CNRS-UEVE, UMR 8587, Laboratoire Analyse et Modélisation pour la Biologie et l'Environnement, Université d'Evry-Val d'Essonne, 91025 Evry, France

\*Corresponding Author :

F. Zal, Station Biologique de Roscoff, Place Georges Teissier, BP74, 29682 Roscoff cedex, FRANCE

26 Phone: 0033 (0)298292309

27 Fax: 0033 (0)298292324

28 E-mail: [zal@sb-roscoff.fr](mailto:zal@sb-roscoff.fr)

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30 Abbreviations: AcNH<sub>4</sub>, ammonium acetate; DPG, D-2,3-diphosphoglycerate; ESI-MS,

31 electrospray ionization mass spectrometry; FA, formic acid; MWC model, Monod-Wyman-

32 Changeux model; SEC, size-exclusion chromatography; TEA, triethanolamine

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34

35 ABSTRACT

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37       The interaction of L-lactate and divalent cations with *Carcinus maenas* hemocyanin has  
38 been probed by electrospray ionization mass spectrometry under conditions preserving non-  
39 covalent interactions (native ESI-MS). *Carcinus maenas* native hemocyanin in the  
40 hemolymph occurs mainly as dodecamers and to a lesser extent as hexamers. A progressive  
41 acidification with formic acid after alkaline dissociation resulted in the preferential  
42 recruitment of the two lightest subunits into light dodecamers, a molecular complex absent  
43 from native hemolymph, in addition to regular dodecamers and hexamers. Addition of  
44 L-lactic acid also induced the recruitment of these subunits, even at alkaline pH. A  
45 dodecamer-specific subunit is needed to enable aggregation over the hexameric state.  
46 Experiments with EDTA suggested the existence of different binding sites and association  
47 constants for divalent cations within hexameric structures and at the interface between two  
48 hexamers. L-lactic acid specific interaction with the lightest subunits was not inhibited by  
49 removal of the divalent cations.

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53 Hemocyanin, the blue respiratory pigment responsible for oxygen transport in decapod  
54 crustaceans, is a macromolecular complex comprised of 75 kDa subunits. These subunits can  
55 each reversibly bind one dioxygen molecule between the two copper ions of their active site  
56 and they associate into non-covalent complexes which can be hexamers (e.g. in isopods, krill,  
57 shrimps, prawns, spiny lobsters), dodecamers (e.g. in brachyurans, lobsters, crayfishes, hermit  
58 crabs) and in some species 24-mers (e.g. in thalassinid shrimps) (Markl, 1986; Markl &  
59 Decker, 1992; Terwilliger, 1998). Hemocyanin circulates as a freely dissolved protein in the  
60 hemolymph of the animals. Its functional properties can be modulated by various effectors  
61 such as  $H^+$ , divalent cations and organic ions (Bridges, 2001; Truchot, 1992). Among them,  
62 the L stereoisomer of lactate is an important allosteric effector produced by the anaerobic  
63 metabolism of the animal, which can be driven by either environmental or metabolic hypoxia  
64 (Truchot, 1980; Truchot & Lallier, 1992). Divalent cations and pH have an effect both on the  
65 structure and on the properties of the complexes since most hemocyanins can be dissociated  
66 under alkaline pH and by dialysis against EDTA (Herskovits, 1988).

67 Arthropod hemocyanins are useful models for studying allosteric regulation  
68 mechanisms. The existence of several types of allosteric effectors and the occurrence of huge  
69 hierarchical quaternary structures in some groups (e.g. 24-mers in arachnids, 48-mers in the  
70 horseshoe crab *Limulus polyphemus* (Markl & Decker, 1992)) allow for investigation of  
71 allosteric mechanisms with different molecular actors and using original models such as  
72 nested allostery (Decker, 1990; Menze et al, 2005). In most decapods, an increase in lactate  
73 concentration increases the oxygen affinity of the pigment (Bridges, 2001; Truchot, 1980;  
74 Truchot, 1992). However, some decapods exhibit low or no sensitivity to lactate (e.g.  
75 thalassinid mud-shrimps (Taylor et al, 2000), the slipper lobster *Scyllarides latus* (Sanna et al,  
76 2004)), notably when the lifestyle becomes more terrestrial (Bridges, 2001; Morris & Bridges,

1994). For one terrestrial species, *Gecarcoidea natalis*, a reverse lactate effect has been observed (Adamczewska & Morris, 1998). The use of chemical analogs and the fact that D-lactate does not affect O<sub>2</sub> affinity suggested that lactate binds stereospecifically to a specific site of hemocyanin (Johnson et al, 1984) and that the binding occurs at all four positions around the chiral carbon (Graham, 1985). For *Callinectes sapidus* hemocyanin, lactate titrations gave values of lactate dissociation constant of 1.8 mM for the oxy-state and 2.2 binding sites per hexamer, and ultrafiltration techniques gave values of 3.2 mM for the dissociation constant and 2.8 sites per hexamer (Johnson et al, 1984). For the spiny lobster *Panulirus interruptus*, purification of each subunit type and reassembly into homohexamers showed that the sensitivity to lactate was subunit-dependent: some homohexamers were sensitive whereas others were not (Johnson et al, 1987). However, in native hexamers as well as in sensitive reassociated homohexamers, analysis of oxygen equilibrium curves showed that there was approximately only one binding site per hexamer, evidencing that the specific site was not located on a single subunit but was formed within the quaternary structure of the protein (Johnson et al, 1987). For *Homarus vulgaris*, ultrafiltration and equilibrium dialysis using labelled ligands showed that there were approximately 2 molecules bound per dodecamer, which is consistent with the finding of one site per hexamer for *Panulirus interruptus*; the same result was observed for urate but urate and lactate binding sites were different and independent (Nies et al, 1992). Moreover, lactate does not bind to hemocyanin in a cooperative way, suggesting that the conformational changes induced by fixation on one site does not influence the other site (Nies et al, 1992). However, a small angle X-ray scattering study (SAXS) of *Homarus americanus* hemocyanin along with electron microscopy showed that upon addition of 10 mM lactate the two hexamers constituting the dodecamer got closer by about 0.5 nm (Hartmann et al, 2001), thus implying that even local changes at the binding sites may have a global effect on the quaternary structure of the protein. Isothermal

titration calorimetry performed on sensible and insensible hemocyanins revealed that insensible hemocyanins did not bind lactate, whereas the sensible ones did (Taylor et al, 2000). A recent study of O<sub>2</sub>-dissociation curves of *Carcinus maenas* hemocyanin reported the binding of 0.35 lactate ion per functional subunit, that is to say 4 sites per dodecamer or 2 sites per hexamer (Weber et al, 2008). This is different from the data from *Homarus vulgaris* and *Panulirus interruptus* but close to the results for *Callinectes sapidus*, a brachyuran crab like *Carcinus*. In this context, the precise mechanism of lactate binding, the involved amino-acids and the induced conformational changes remained to be determined.

Many studies have also demonstrated the crucial role of calcium and pH for hemocyanin structure and function. Upon dialysis against EDTA to remove calcium and pH increase, complexes are dissociated and O<sub>2</sub>-binding capacity is lost (Brenowitz et al, 1983; Herskovits, 1988; Molon et al, 2000; Olianias et al, 2006). Some species show unusual stability against dissociation (Beltramini et al, 2005). Usually, reassociation occurs upon return to physiological pH values and addition of calcium. Divalent cations and pH are also known to modify hemocyanin oxygen-binding properties (Andersson et al, 1982; Bridges, 2001; deFur et al, 1990; Morimoto & Kegeles, 1971; Olianias et al, 2006; Truchot, 1992).

In recent years, mass spectrometry (MS) has emerged as a new and potent tool for structural analysis of protein complexes and protein interactions with various ligands. The possibility of preserving the fragile non-covalent interactions through the electrospray ionization (ESI) process has allowed probing quaternary structures of various assemblies and following association and dissociation of non-covalent complexes and incorporation of small organic molecules or inorganic ions (Potier et al, 1997; Rogniaux et al, 2001; Tahallah et al, 2002; van Duijn et al, 2006). The ESI source was also successfully used to study the quaternary structure of high-molecular mass invertebrate respiratory pigments (Bruneaux et

al, 2008; Green et al, 2001; Green et al, 1998; Green & Vinogradov, 2004; Sanglier et al, 2003; Zal et al, 2002).

ESI-MS ability to characterize compounds by their mass with a high precision and to maintain non-covalent interactions such as exist between hemocyanin and L-lactate and divalent cations makes this technique a useful tool to probe the specificity of these interactions and their effect on the quaternary structure of the complex. For *C. maenas*, hemocyanin is the major protein found in the hemolymph and occurs mainly as dodecamers and to a lesser extent as hexamers (typically 80 to 95 % dodecamers in mass). Increases of pH, L-lactate concentration or divalent cations concentration are known to increase its oxygen affinity (Truchot, 1975; Truchot, 1980). Its constituting subunits were characterized by ESI-MS in denaturing conditions and the complexes by ESI-MS in native conditions, as described in the study by Sanglier and collaborators (Sanglier et al, 2003). In this paper, we expose an attempt to dissociate the subunits at alkaline pH and to probe their potential association with L-lactate in non-covalent conditions. An investigation of the influence of divalent cations on the reassociation of subunits was also performed using EDTA.

## MATERIAL AND METHODS

### Animal collection and hemocyanin sampling and purification

*Carcinus maenas* individuals were caught by baited traps in the intertidal zone in Roscoff, France, and kept in running sea water at ambient temperature (salinity 35-35.4 ‰, temperature 15-17.5°C). Hemolymph was withdrawn with a syringe through the articular membrane of a walking leg and immediately put on ice. Samples were centrifugated for 10 min at 10000 g to pellet cells and coagulated proteins. For whole hemolymph study, the supernatant was recovered and frozen at -20°C. For purified complexes study, dodecamers



and hexamers were separated by size-exclusion chromatography (SEC) using a Superose-6 10/300 GL column (Amersham Bioscience) at an elution rate of 0.25 ml/min with a crustacean physiological saline buffer (500 mM NaCl, 10 mM KCl, 30 mM MgSO<sub>4</sub>, 20 mM CaCl<sub>2</sub>, 50 mM Tris, pH 7.8, modified from (Chausson et al, 2004)). Collected fractions purity was assessed with the same system. Purified samples were frozen at -20°C.

For preliminary analyses of hemocyanin in denaturing and non-covalent conditions, samples were withdrawn from several individuals kept in running sea water under several salinity and oxygenation conditions. For all dissociation and association experiments using triethanolamine (TEA), formic acid (FA), L-lactic acid and EDTA, the whole-hemolymph samples were issued from the same individual and the purified complexes were issued from another single individual; those samples were withdrawn one day after catching the crabs.

#### Sample preparation for ESI-MS

All samples used for ESI-MS were desalted through concentration-dilution cycles using 10 kDa Microcon (Millipore) prior to analysis. Typically, 30 µl of native hemolymph diluted in 470 µl 10 mM ammonium acetate (AcNH<sub>4</sub>), pH 6.8 or 250 µl of purified sample (protein concentration about 1-3 µg/µl) were first concentrated to almost dryness and then resuspended in 400 µl 10 mM AcNH<sub>4</sub>. At least 10 successive similar concentration-dilution steps were performed in order to ensure that the samples were correctly desalted prior to ESI-MS analysis. The samples were stored at 4°C in 10 mM AcNH<sub>4</sub> until analysis. The desalting was realised just prior to MS analysis and desalted samples for non-covalent analysis were not kept more than 2 days at 4°C because of dissociation occurring upon longer storage.

#### Non-covalent and denaturing ESI-MS

Mass spectrometry experiences were performed on a MicroTOF instrument (BRUKER Daltonics, Bremen, Germany) equipped with an ESI source.

For non-covalent analysis, desalted samples were diluted in 10 mM AcNH<sub>4</sub> at a final concentration of approximately 1.5 μM (for 900 kDa dodecamers). The injection rate for the samples was 4 μl/min. The ESI needle voltage was set to 5 kV, nebulization gas (N<sub>2</sub>) pressure was 1 bar, drying gas flow was 4 l/min, source temperature was 200°C and the capillary exit voltage was set to 400 V. The calibration was made using 1 mg/ml CsI in water/isopropanol (50:50 volume). The acquisition range was 500 to 20000 m/z. Spectra were smoothed using a Savitzky-Golay method and baseline subtracted. Complexes masses were estimated using a built-in ruler (BRUKER DataAnalysis v3.2 software).

For denaturing analysis, desalted samples were diluted in a water/acetonitrile/formic acid mix (H<sub>2</sub>O/ACN/FA 50:50:1 volume) at a final concentration of approximately 4 μM (for 75 kDa subunits). The nebulization gas (N<sub>2</sub>) pressure was 0.3 bar, drying gas flow was 3 l/min, and the capillary exit voltage was set to 160 V. Calibration was performed using myoglobin (SIGMA-ALDRICH). Mass spectra were analysed using maximum entropy deconvolution (BRUKER DataAnalysis v3.2 software).

In the following, denaturing analysis refers to ESI-MS analysis in H<sub>2</sub>O/ACN/FA with classical instrumental parameters. In these conditions, all non-covalent interactions are broken. Non-covalent analysis refers to ESI-MS analysis in AcNH<sub>4</sub> with “gentle” instrumental parameters. In this case, non-covalent interactions are preserved during the analysis, even if biochemical treatments can partially dissociate the complexes before analysis.

#### Dissociation in TEA

For alkaline denaturation of hemocyanin, desalted samples were diluted in 10 mM AcNH<sub>4</sub> with various TEA concentrations (from 0.005 % to 0.05 % TEA in volume, pH 7.52 to 9.02 respectively) and incubated for 15 min at ambient temperature before non-covalent ESI-MS analysis was performed.

#### Reassociation with formic acid

For acidic reassociation, desalted hemocyanin was first dissociated in 10 mM AcNH<sub>4</sub>, 0.03 % TEA (pH 8.6) for 15 min at ambient temperature and then formic acid was added to a final concentration of 0.001 % to 0.04 % (pH 8.48 to 4.22, respectively); the preparation was incubated for another 15 min before performing non-covalent ESI-MS analysis to allow for reassociation.

#### Effect of L-lactic acid

For investigation of L-lactic acid effect, desalted hemocyanin was first dissociated in 10 mM AcNH<sub>4</sub>, 0.03 % TEA (pH 8.6) for 15 min at ambient temperature and then lactic acid was added to a final concentration of 2 mM (L(+)-lactic acid, SIGMA-ALDRICH), along with TEA to counterbalance the acidifying effect of lactic acid. After addition, the final TEA concentration ranged from the original 0.03% to 0.07%, and pH values ranged from 5.89 to 8.58. The preparation was left to incubate for 15 min at ambient temperature before non-covalent ESI-MS analysis.

#### Effect of chelation of divalent cations by EDTA

Divalent cations were removed using EDTA in order to investigate their structural role. Two 10 mM Na<sup>+</sup>-EDTA, 10 mM AcNH<sub>4</sub> solutions were prepared with different pH, one with 0.11 % TEA (approximative pH 7.5) and the other with 0.15 % TEA (approximative pH 9). A

desalted total hemolymph sample was diluted 20-fold in each of these solutions, concentrated on a 10 kDa Microcon filter, diluted in the same EDTA mix again and then washed by 6 concentration-dilution steps in 10 mM AcNH<sub>4</sub>, 0.005 % TEA and 0.05 % TEA respectively in order to remove the Na<sup>+</sup> and EDTA salts along with the chelated cations. Non-covalent analysis was performed after this step. Then, L-lactic acid was added up to 2 mM final concentration with TEA to adjust pH either at 7.5 or 9 approximately and the preparation was left to incubate for 15 min at ambient temperature before analysis.

## RESULTS

### Preliminary analysis of *Carcinus maenas* hemocyanin

28 different hemolymph samples from 14 different male individuals issued from control or acclimation experiments were analysed by ESI-MS in denaturing conditions. Six main subunits were most frequently observed, of which one was only observed in the dodecameric fraction when analyses were performed on SEC-separated, purified complexes. No covalently bound dimer was ever observed. Table I presents the masses obtained in our study for these subunits and the nearest masses obtained in a previous study by Sanglier and collaborators (Sanglier et al, 2003). In this previous study, a total of nine subunits were identified by the authors with four of them exhibiting low intensity peaks, the other five being identical to the subunits identified in our study.

Hemocyanin analysis by ESI-MS in non-covalent conditions produced two main charge-state distributions corresponding to dodecameric and hexameric species (figure 1). The observed dodecamer to hexamer ratio is similar to the one determined by SEC; this confirms that the desalting steps do not markedly alter the oligomerization equilibrium provided that the analysis is made just after desalting (storage shorter than 2 days at 4°C). Some high-mass

distributions can also be observed with a lower intensity, corresponding to 18-meric and 24-meric complexes (up to 13 and 7 %, respectively). SEC experiments coupled with light-scattering mass determination (not presented here – personal observation) also revealed the presence of a slight amount of these high molecular-mass complexes.

#### Alkaline dissociation of complexes by TEA and acidic reassociation by formic acid

A native hemolymph sample was progressively dissociated to its constituting subunits by adding increasing quantities of TEA (figure 2). Before addition of TEA, one main species is observed and corresponds to a 900 kDa mass (dodecamer, figure 2 panel A). A less intense signal is visible corresponding to a hexamer mass around 450 kDa and a slight monomer signal is also visible. It can be noted that the particular native sample shown here contains almost only dodecamers and only very few hexamers. Experiments on SEC-purified dodecamers and hexamers are detailed later. Upon TEA addition, the intensity of the dodecamer distribution decreases while the monomers peaks get more intense (figure 2 panels B,C). A second distribution with higher  $m/z$  values and lower charge states is observed; precise charge-state assignment is difficult for this due to the high mass of the complex. This distribution can correspond either to an 885 kDa or to an 864 kDa mass. Given that no subunit of  $864/12 = 72$  kDa is observed in denaturing conditions, this distribution can be identified unambiguously with the 885 kDa mass. This mass fits well with a dodecamer reassociated from the lightest subunits (below 74 kDa) produced by alkaline dissociation. The occurrence of this species before acidic reassociation suggests that a dynamic equilibrium between it and the free subunits exists as soon as dissociation of the dodecamers begins. Relative abundance of residual dodecamer after alkaline dissociation could vary slightly from one experiment to the other, but a massive dissociation was always observed.

The presence of dissociated subunits at various stages of the acidification process can be monitored by examining the distribution around  $m/z$  4400, corresponding to the monomers with a 17  $H^+$  charge (figure 2 panels F,G,H). Six main charge-state distributions are visible, corresponding to six subunits. The estimated masses are depicted in table I. They could vary slightly from one experiment to another, but were almost always superior to the masses observed in denaturing conditions. Interestingly, bimodal charge state distributions are observed for monomers when pH increases (figure 2 panel C). These distributions suggest that different conformational states exist for the dissociated monomers.

After alkaline dissociation of the complexes into their subunits, the preparation is acidified by progressive addition of formic acid (figure 2 panel D,E). When pH decreases from 9 to 7.7, a progressive and partial reassociation into complexes is observed, mainly into light dodecamer (dl, 885 kDa) and dodecamer (d, 900 kDa) complexes (figure 2 panel F). In the meantime, a progressive disappearance of the Cm1 and Cm2 peaks corresponding to the lightest monomers suggests that these subunits are preferentially recruited for reassociation into complexes (figure 2 panel H). When pH becomes strongly acidic (below the isoelectric point), complexes are dissociated anew and all subunits peaks are visible again (data not shown).

The same experiments were performed using purified dodecamers and purified hexamers. Whereas dodecamers d and light dodecamers dl were obtained from dissociated monomers issued from purified dodecamers, only hexamers and no dodecamers (d or dl) were obtained from dissociated monomers issued from purified hexamers, indicating that the dodecamer-specific subunit Cm6 is compulsory for the association into dodecamers (results not shown).

Reassociation by L-lactic acid

L-lactic acid also induced a partial reassociation of the subunits into complexes, as observed with formic acid (figure 3). The main complex observed is the light dodecamer, with a relative intensity higher than in the case of formic acid reassociation (figure 3 panel B). A preferential mobilization of the lightest subunits is observed again, which is consistent with the appearance of light dodecamer (figure 3 panels E,F). While this preferential recruitment is also observed with formic acid, there is an important difference since L-lactic acid induces an immediate disappearance of the two light subunits (Cm1 and Cm2) as soon as it is added, even if the pH is maintained at a dissociating value over 8.4. This specific and immediate effect contrasts with the progressive effect observed with formic acid. The bimodal charge state distributions of the dissociated monomers is still observed in these conditions. Cm1 and Cm2 remain undetectable at pH 5.9, over the isoelectric point.

#### Chelation of divalent cations by EDTA

When divalent cations are removed by EDTA washing after alkaline dissociation of whole hemolymph preparation, returning to pH 7.5 by washing with a 10 mM AcNH<sub>4</sub>, 0.005 % TEA solution induces a partial reassociation mainly into hexamers and to a lesser extent into dodecamers (figure 4 panel B). Interestingly, all dissociated monomers peaks disappear upon reassociation except for the 4450 peak corresponding to the subunit specific of the dodecameric assembly Cm6 (figure 4 panel F); the high proportion of hexamers formed can be explained by the limited incorporation of Cm6 in the reassociated complexes. Addition of L-lactic acid to the sample after removal of EDTA still resulted in the disappearance of the lightest subunits peaks (figure 4 panels G,H) but hexamers remained the dominant complexes and no light dodecamer were observed (figure 4 panels C,D). No major dissociation into hexamers or monomers was observed upon addition of EDTA at neutral pH (data not shown).

## DISCUSSION

### Subunits masses in denaturing and non-covalent conditions

The masses observed in denaturing conditions are in good agreement with those previously published by Sanglier and collaborators, also in denaturing conditions (Sanglier et al, 2003). The five most abundant subunits observed in Sanglier's study are part of the six subunits observed here.

In our study, a slight and variable difference is observed between masses estimated from denaturing and from non-covalent conditions. The higher masses estimated in non-covalent conditions can be accounted for by the occurrence of various adducts to the dissociated subunits in these conditions, such as alkaline ions, divalent cations or unremoved copper ions in the active site. These adducts are permitted by the unusual conditions used here, namely an alkaline dissociation observed in ESI-MS conditions preserving the remaining non-covalent interactions during the analysis. Desolvation conditions also differ between ESI-MS in denaturing and in non-covalent conditions, hence the amount of the remaining solvent is likely to be different.

### Complexes masses and abundances in non-covalent conditions

The use of ESI-MS in non-covalent conditions enables to acquire signal for high-molecular mass non-covalent complexes in their native state (dodecamers and hexamers). The good agreement between dodecamer to hexamer ratios from ESI-MS and SEC confirms that our ESI-MS data under near-native conditions are relevant for studying complexes occurring in solution (figure 1). For occurrence of 18-mer and 24-mer, aggregation is known to be possible during the ESI process but a slight fraction of 18-mer and 24-mer is also visible by SEC coupled with a light-scattering system (personal observation for *Carcinus maenas*



hemocyanin; (Beltramini et al, 2005) for *Penaeus monodon* hemocyanin). However, 24-mers can be detected from purified 12-mers (personal observation). This is unlikely to come from a contamination during the purification step since no 18-mer is visible, but must be due to aggregation of the sample during preparation or during the ESI-MS process. Whatever the extent of aggregation process is for these high-mass complexes (18-mer and 24-mer), their concentration is always very low compared to dodecamer and hexamer. Even if they may really exist in the hemolymph of the animal and not be formed during the sampling and storage, they are probably of very little physiological significance due to this low concentration.

#### Alkaline dissociation and reassociation (native and purified)

Crustacean hemocyanin dissociation is known to be induced by high or low pH (Herskovits, 1988), with varying stability ranges depending on the species: for example, contrary to other crustacean hemocyanins, *Penaeus monodon* hemocyanin has been shown to have a high stability against dissociation at high pH and in the presence of EDTA (Beltramini et al, 2005). For other crustaceans, various experiments have shown that alkaline dissociation was reversible and that complexes could be reassociated from whole subunit sets or from selected purified subunits (Dainese et al, 1998; Johnson et al, 1987). In our study the dissociation is induced by alkaline pH and yields separated subunits from the complexes. The dissociation efficiency could vary from one experiment to another, and part of the complex could resist the dissociation; however most of the time the dissociation was almost complete, showing that ESI-MS can be used to monitor a process taking place in solution and that the different aggregation states present in the aqueous phase are likely to be conserved during the ionization and analysis processes. Previous studies (Chantler et al, 1973; Dainese et al, 1998)

also showed the dissociating effect of EDTA and alkaline pH on a close species (*Carcinus aestuarii* also named *Carcinus mediterraneus*).

The occurrence of bimodal charge state distribution for dissociated monomers must correspond to different conformational states of the monomers. These distributions appear at high pH and bimodal distributions can correspond to partial unfolding of the subunits coupled to dissociation. However, unfolding is usually associated with a shift from narrow distributions of low charge-density ions towards broader distributions of higher charge-density ions, reflecting that the unfolded proteins are less structured and can accommodate more charges on their surface (Kaltashov 2008). In our study, lower charge-density ions appear at high pH which is in contradiction with what is expected from the unfolding model. Moreover, the occurring process must be reversible since the bimodal distributions almost disappear when pH decreases again (figure 2, panel E). Many other processes occurring in the gas-phase can also alter the charge state distribution (Kaltashov 2008) and in our case the significance of the observed bimodal distributions remains unclear. A systematic study of hemocyanin denaturation coupling ESI-MS analysis and circular dichroism would help to resolve this issue, as already performed by another group with hemoglobin (Griffith 2003).

The reassociation by returning to a more neutral pH could also be monitored by ESI-MS. Complete reassociation was never observed and the fact that the lightest subunits are reassociated first, as evidenced by the monomers peaks and by the occurrence of the light dodecamer, shows that the reassociation process after dissociation is not exactly identical to the one occurring naturally. A dynamic equilibrium between subunits and light dodecamer is also observed (figure 2 panel B). In several species, homohexamers could be reassociated from purified subunits but reassociation into dodecamers necessitated the occurrence of specific subunit types (Stöcker et al, 1988). However, it was suggested in a study of *Paralithodes camtschaticae* hemocyanin that homododecamers could be formed for this

species but this case is original because dodecamers and hexamers seem to exist in a chemical equilibrium (Molon et al, 2000). Here we cannot distinguish if two light homododecamers are produced since the distributions would overlay, but the fact that the purified hexamers cannot reassociate into dodecamers evidences that the dodecamer specific subunit is compulsory to form dodecamers. Hence, we should have a simultaneous association of the light subunits and of the dodecamer-specific subunit when reassociating dodecamers from alkaline dissociation. This implies different association constants and different interactions between subunit types.

#### Specific effect of L-lactate

L-lactic acid addition has the same effect that acidification by formic acid since the same light subunits are mobilized first and light dodecamers are formed first. However, the fact that lactic acid has an effect even at a dissociating high pH where formic acid has no effect yet shows a specific effect of the molecule. Moreover, it triggers the formation of light dodecamers much more than classic dodecamers, enabling a specific stabilization/association of this form. Figure 5 is a schematic summary of the steps involved in the dissociation and reassociation studied here. Based on the previously published data suggesting the occurrence of a site emerging from the association of several subunits and the specificity of some subunits for lactate sensitivity in *Panulirus interruptus* (Johnson et al, 1987), it can be hypothesized that these two lactate-sensitive subunits interact together to form a lactate-binding site within the quaternary structure of the dodecamer. Another possibility is that each of the lactate-sensitive subunit is self-sufficient for the formation of a lactate binding site, and that two types of sites involving one or the other light subunit exist within the quaternary structure of the dodecamer. The interaction of L-lactate with the binding site could stabilize the subunits association and hence explain the complexes formation even at high pH (figure 5). For *Carcinus maenas*, 4 such lactate binding sites exist per dodecamer according to Weber

and collaborators (Weber et al, 2008). Since L-lactate can still promote the incorporation of the light subunits Cm1 and Cm2 after washing with EDTA but mainly hexamers are observed in this case, the binding sites must be located within the hexameric structure but not at the interface between two hexamers. However our data are not of a type helping to resolve the number of binding sites per dodecamer. As noted by Graham and collaborators (Graham, 1985), the existence of a binding site arising from the quaternary structure of the complex and not from the tertiary structure of a single subunit would be similar to the organophosphate binding site of vertebrate tetrameric hemoglobin.

#### Effect of chelation of divalent cations

Divalent cations are known to have both functional and structural roles in hemocyanins (Bridges, 2001; Truchot, 1975). Here, the reassociation of subunits into complexes is still possible after alkaline dissociation and chelation of the divalent cations by EDTA but the reassociation tends to stop at the hexamer level, and at least part of the dodecamer-specific subunit remains unassociated. The incorporation of the specific subunit must involve lower association constants or need more cations than the rest of the assembly process since  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are removed or at least severely depleted upon chelation by EDTA. The dodecamer formation process can be limited either by lower incorporation of the specific subunit in hexameric assemblies or by a lower rate of hexamers association into dodecamers even with the specific subunit incorporated within the hexameric structure. As underlined by Stöcker and collaborators, the inter-hexamer link varies between species: it can be an ionic bond with divalent cations as for *Homarus americanus* or a disulfide linked dimer as for *Astacus leptodactylus* and *Cherax destructor* (Markl & Decker, 1992; Marlborough et al, 1981; Stöcker et al, 1988). The same group also suggested that subunits could substitute during the hexamer formation due to homologous interaction sites between them whereas some specific

interactions between precise subunits could be needed to form the dodecamer, hence explaining the reassociation limited to hexamers. In *Carcinus maenas* hemocyanin, the interactions between hexamers must be ionic since no covalently-bound dimer can be found. The study of the hemocyanin of *Paralithodes camtschaticae* by Molon and collaborators (Molon et al, 2000) also showed that after dialysis against EDTA, dodecamers were dissociated into hexamers. However, the hemocyanin of this anomuran exists as a chemical equilibrium between the two forms (dodecamers and hexamers) in native conditions and homododecamers and homo-hexamers can be formed from one purified subunit type. In this case, one type of monomer is able to form all the interactions needed to form both forms. The fact that dissociated hexamers cannot reassociate into dodecamers in our study implies that for *Carcinus* only some specific monomers are able to establish the interactions needed for the dodecamer assembly.

Typically, two different types of calcium-binding sites with high and low affinities are observed for crustacean hemocyanins (table II) (Andersson et al, 1982; Molon et al, 2000; Sanna et al, 2004) and in the case of Andersson's study  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were shown to have similar affinities for the binding sites. There are usually a few sites with high affinity (1 to 3 sites per hexamer) and numerous with a 10 to 100-fold lower affinity (1.6 to 42 sites per hexamer). Given the physiological range of calcium concentration, the sites with the highest affinity are always saturated and would rather play a structural role while the sites with the lowest affinity would have a modulating effect on oxygen affinity since their saturation can vary depending on the calcium concentration in the hemolymph (Andersson et al, 1982; Johnson et al, 1988). It is likely that the structural calcium and magnesium ions bound to the high-affinity sites are retained throughout the desalting process while the others divalent cations and  $\text{Na}^+$  ions are removed (consistent with a 100-fold lower affinity of hemocyanin for  $\text{Na}^+$  (Andersson et al, 1982)). Since no EDTA effect is observed here without prior

alkaline dissociation, these sites must be located at the interface between different subunits (Hazes et al, 1993) and be accessible to chelation by EDTA only after separation of the subunits. The reassociation into hexamers rather than dodecamers after removal of the structural cations can indicate that less cationic bridges are needed between subunits within a single hexamer than between two hexamers implicated in a dodecamer. Another possibility is the existence of some intra-hexamer sites which would retain divalent cations with a very high affinity. The fact that the specific effect of L-lactate is not inhibited by EDTA shows that no low-affinity bound divalent cations are necessary for the direct interaction between lactate and hemocyanin.

#### Structure of the L-lactate binding site

Crustacean hemocyanin is a very complete model for the study of structural and functional properties of respiratory pigments and more generally allosteric proteins. The multimeric structure made of functionally different yet similar subunits and the diversity of effectors and of their effect allow for numerous biochemical issues to be addressed. Here, we used non-covalent ESI-MS to probe the structural effects of L-lactate and divalent cations on *Carcinus maenas* hemocyanin. The specific interaction of L-lactate with 2 subunits and its stabilizing effect have been evidenced, as well as the role of divalent cations for multimeric assembly. The question of the precise structure of the binding site of L-lactate remains to be solved. The specificity of interaction with some subunits, the symmetry of the quaternary structure and the L-lactate asymmetry are to be considered (Johnson et al, 1984). The fact that sensitive homohexamers only harbor one site and that the number of observed sites per hexamer is about two for brachyuran crabs suggests that the sites are located on the three-fold axis of the hexamer, and hence possess the same symmetry. How does the binding between such a symmetric site and a chiral ligand occur? The site may present three potential positions

for lactate binding and the binding of one molecule on one site would prevent the binding at the other sites by steric hindrance. Another possibility is that the binding site is stabilized in an asymmetric conformation when L-lactate binds to it. Arnone showed for human hemoglobin that the crystallographic map of the protein with the asymmetric ligand D-2,3-diphosphoglycerate (DPG) showed the same non-crystallographic dyad axis as the map for the protein alone, and deduced that the binding of DPG occurred in two symmetric orientations related by a 180° rotation (Arnone, 1972). More recent studies with lower-salt crystals or using <sup>31</sup>P nuclear magnetic resonance in solution showed that the binding site of DPG was actually asymmetric in the presence of the ligand (Pomponi et al, 2000; Richard et al, 1993). Molecular dynamics simulations also suggested that a dynamic heterogeneity existed in the hemoglobin tetramer and that DPG influences the tertiary states explored by the protein (Laberge & Yonetani, 2008). A similar mechanism can be postulated for hemocyanin, with the occurrence of a dynamic heterogeneity influenced by the presence of effectors such as oxygen and lactate, resulting in a stabilized asymmetric binding site when L-lactate is effectively bound. Performing studies similar to those made on hemoglobin and DPG with hemocyanin and L-lactate would help to test this hypothesis.

It has been showed that the oxygenated Hc hexamer of *Panulirus interruptus* has a reduced channel along the three-fold axis compared to the deoxygenated form (De Haas et al, 1993). Using the simple Monod-Wyman-Changeux (MWC) model for *Carcinus maenas* Hc dodecamer, Weber and collaborators showed that L-lactate increases O<sub>2</sub> affinity in part by shifting the allosteric equilibrium towards the high-affinity R state (Weber et al, 2008). From these data it can be suggested that the potential binding of lactate in the reduced central channel could stabilize the oxygenated R conformation. Such a hypothesis must be considered with care and at the hexamer scale, since SAXS studies of the Hc dodecamer of *Homarus americanus* have showed that the oxygenated dodecamer exists in two different forms, the

one without lactate and the one in the presence of lactate with the two hexamers closer by 0.5 nm. In this case the simple MWC model is no longer relevant and nested allostery must be considered (Hartmann et al, 2001).

It would be of interest to perform studies similar to those conducted here using hemocyanin from terrestrial crabs or mud shrimps with little or no lactate effect, and to test the effect of other molecules such as the stereoisomer D-lactate or the physiological effector urate.

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## FIGURE LEGENDS

**Figure 1:** Mass spectrum of *Carcinus maenas* native hemolymph analysed under non-covalent conditions (pH 6.8). Spectra were acquired using individual whole hemolymph samples. Aggregation state, estimated mass and m/z value for the main peak of each distribution are indicated. Insert: size-exclusion chromatography profile of the same sample with a dodecamer-to-hexamer ratio of 9:1.

**Figure 2:** Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin by TEA and formic acid. Panels A to E, mass spectra of a whole hemolymph sample desalted in 10 mM AcNH<sub>4</sub> with increasing quantities of TEA (0.005, 0.03 and 0.05 % TEA for pH 7.6, 8.6 and 9, respectively) then formic acid (0.004 and 0.0045 % formic acid with 0.03 % TEA for pH 7.9 and 7.7, respectively). m, monomers peaks, h, hexamer peaks (450 kDa), d, dodecamer peaks (900 kDa), dl, light dodecamer peaks (885 kDa). Panels F to H, focus for each mass spectrum on the monomer peak distributions in the 4300-4600 m/z range, in which the 17<sup>+</sup> charged peak of each monomer is expected. Six overlapping distributions can be observed. The presence or absence of each dissociated subunit can be determined by examining the peaks visible in this m/z range. Masses obtained in non-covalent and denaturing conditions for each subunit are compared in table I.

**Figure 3:** Acidic reassociation of *Carcinus maenas* hemocyanin by L-lactic acid. Panel A, mass spectrum of a whole hemolymph sample dissociated in 10 mM AcNH<sub>4</sub>, 0.03 % TEA, pH 8.6. Panels B and C, mass spectra of the dissociated hemocyanin treated with 2mM lactic acid and with or without addition of a further 0.04 % TEA (pH 8.6 and 5.9, respectively). m, monomers peaks, h, hexamer peaks (450 kDa), d, dodecamer peaks (900 kDa), dl, light

dodecamer peaks (885 kDa). Panels D to F, focus for each mass spectrum on the 4300-4600 m/z range (monomer peaks).

**Figure 4:** Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin coupled with chelation of divalent cations by EDTA. Whole hemolymph sample was washed by an EDTA mix (pH 9) as explained in the materials and methods section and salts were then removed by washing with 10 mM AcNH<sub>4</sub> containing TEA at various concentrations. Panels A to D, whole mass spectra, panels E to H, focus in the monomer peaks m/z range. Sample preparation was as follow: panels A and E, washing with 0.05 % TEA (pH 9); panels B and F, washing with 0.005 % TEA (pH 7.5); panels C and G, washing with 0.05 % TEA and addition of 2 mM lactic acid (pH 8.1); panels D and H, washing with 0.005 % TEA and addition of 2 mM lactic acid (pH 7.4). m, monomers peaks, h, hexamer peaks (450 kDa), d, dodecamer peaks (900 kDa), dl, light dodecamer peaks (885 kDa). Panel C (pH 8.1, 2mM lactate), the hexamer peaks could correspond to a classical hexamer and to a “light” hexamer.

**Figure 5:** Summary of the dissociation and association steps observed under alkalinization by TEA and acidification by formic acid or addition of L-lactate. In the native state before dissociation, the subunit composition is unknown but two Cm6 subunits must interact to form the dodecamer. Upon progressive dissociation by alkalinization, dissociated monomers can partially reassociate into light dodecamers by dynamic equilibrium. Monomers are fully dissociated at pH 9 and can be reassociated either by adding L-lactic acid even when maintaining a high pH or by progressive acidification by formic acid. L-lactic acid immediately promotes the reassociation of light subunits into light dodecamer and must interact with hemocyanin at two sites per hexamer, located on both sides of the three-fold axis

789 of each hexamer, at the center of each trimer. Note that partial unfolding of the dissociated  
790 monomers could occur (not figured here – see the text for details).

791

792 (references cited in the tables: (Andersson et al, 1982; Brouwer et al, 1983; Johnson et al,  
793 1988; Kuiper et al, 1979; Sanna et al, 2004))

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Table I: subunit masses for *Carcinus maenas* hemocyanin obtained by ESI-MS

Subunit name	Mass obtained by Sanglier et al. 2003 (Da) <sup>a</sup>	Average mass in denaturing ESI-MS (Da $\pm$ s.d.) <sup>b</sup>	m/z value for the (17 H <sup>+</sup> ) peak	Examples of masses estimated by non-covalent ESI-MS (Da) <sup>c</sup>		Mass differences between non-covalent and denaturing ESI-MS (Da) <sup>d</sup>	
				no.1	no.2	no.1	no.2
Cm1	73931	73922 $\pm$ 1.3	4360	74116	74090	194	168
Cm2	74049	74043 $\pm$ 1.1	4368	74218	74207	175	164
Cm3	75088	75073 $\pm$ 3.2	4418	75106	75089	33	16
Cm4	75161	75187 $\pm$ 5.8	4426	75253	75214	66	27
Cm5	75234	75224 $\pm$ 1.8	4435	75344	75355	120	131
Cm6*	75459	75449 $\pm$ 0.9	4450	75684	75629	235	180

\*Dodecamer specific subunit

<sup>a</sup>Nine masses were determined by Sanglier and collaborators<sup>38</sup>; the masses that were the closest to those we determined are reported here

<sup>b</sup>Average masses and standard deviations are calculated from data for 14 different individuals; the number of values for each subunit varies from 9 to 35 depending on the occurrence of each subunit in different individuals and in purified fractions for each individual

<sup>c</sup>These masses were calculated from the monomers charge-state distributions from two different experiments

<sup>d</sup>These differences were calculated using the average masses determined in denaturing conditions in this study; molecular masses of potential adducts in non-covalent conditions are for example 127.1Da (the two Cu of the active site), 23Da (Na), 24.3Da (Mg), 40.1Da (Ca) and combinations thereof

Table II: association constants and number of sites for calcium binding with hemocyanin

Species	High-affinity sites		Low-affinity sites		pH	References
	$K_{Ca} (M^{-1})$	number of sites per hexamer	$K_{Ca} (M^{-1})$	number of sites per hexamer		
<i>Panulirus interruptus</i>	$1 \times 10^4$		50		7.6	(51)
<i>Panulirus interruptus</i>	$3 \times 10^4$	1	$1.5 \times 10^3$	3-17	7.0	(26)
<i>Callinectes sapidus</i>	$4-9.1 \times 10^4$	2.4-3	$2-10 \times 10^2$	14-42		(52)
<i>Callinectes sapidus</i>			$7.1 \times 10^2$	1.6-2 <sup>a</sup>	7.01	(44)
			$3.3 \times 10^2$	3.5-5.4 <sup>a</sup>	7.55	
<i>Scyllarides latus</i>	$5.6 \times 10^4$		1.3		7.0	(12)

<sup>a</sup>The number of sites calculated in this study corresponds only to oxygen-linked binding sites and not to the total number of binding sites; the difference with results from (52) led the authors to the conclusion that many of the calcium binding sites did not affect oxygen binding

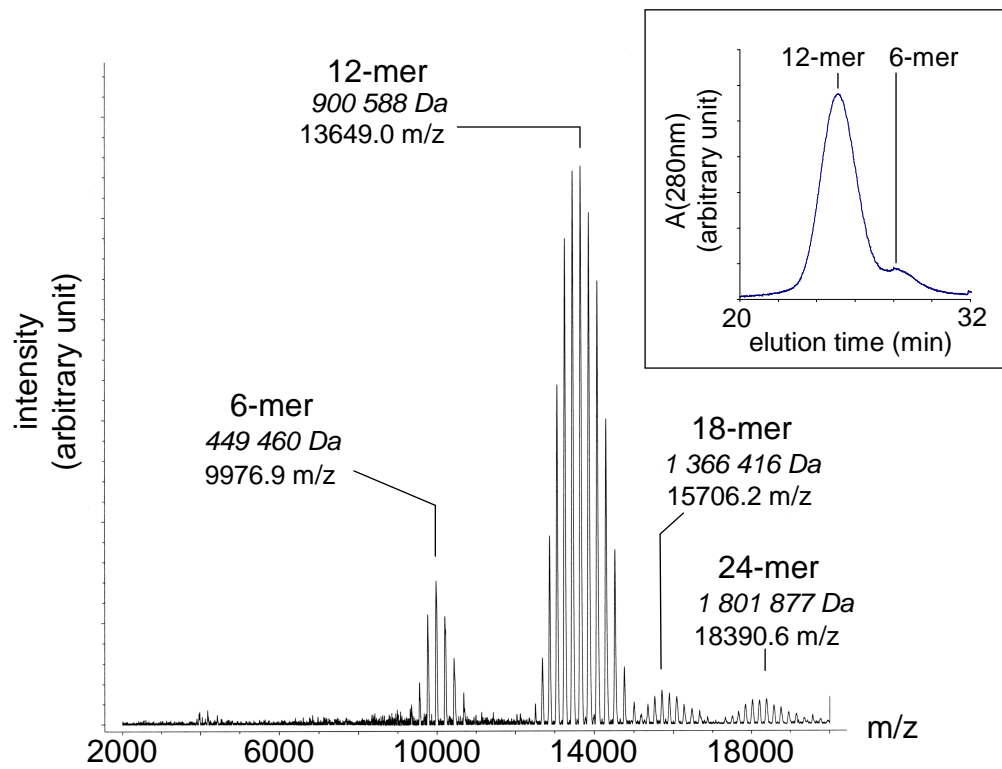


Figure 1: Mass spectrum of *Carcinus maenas* native hemolymph analysed under non-covalent conditions (pH 6.8)

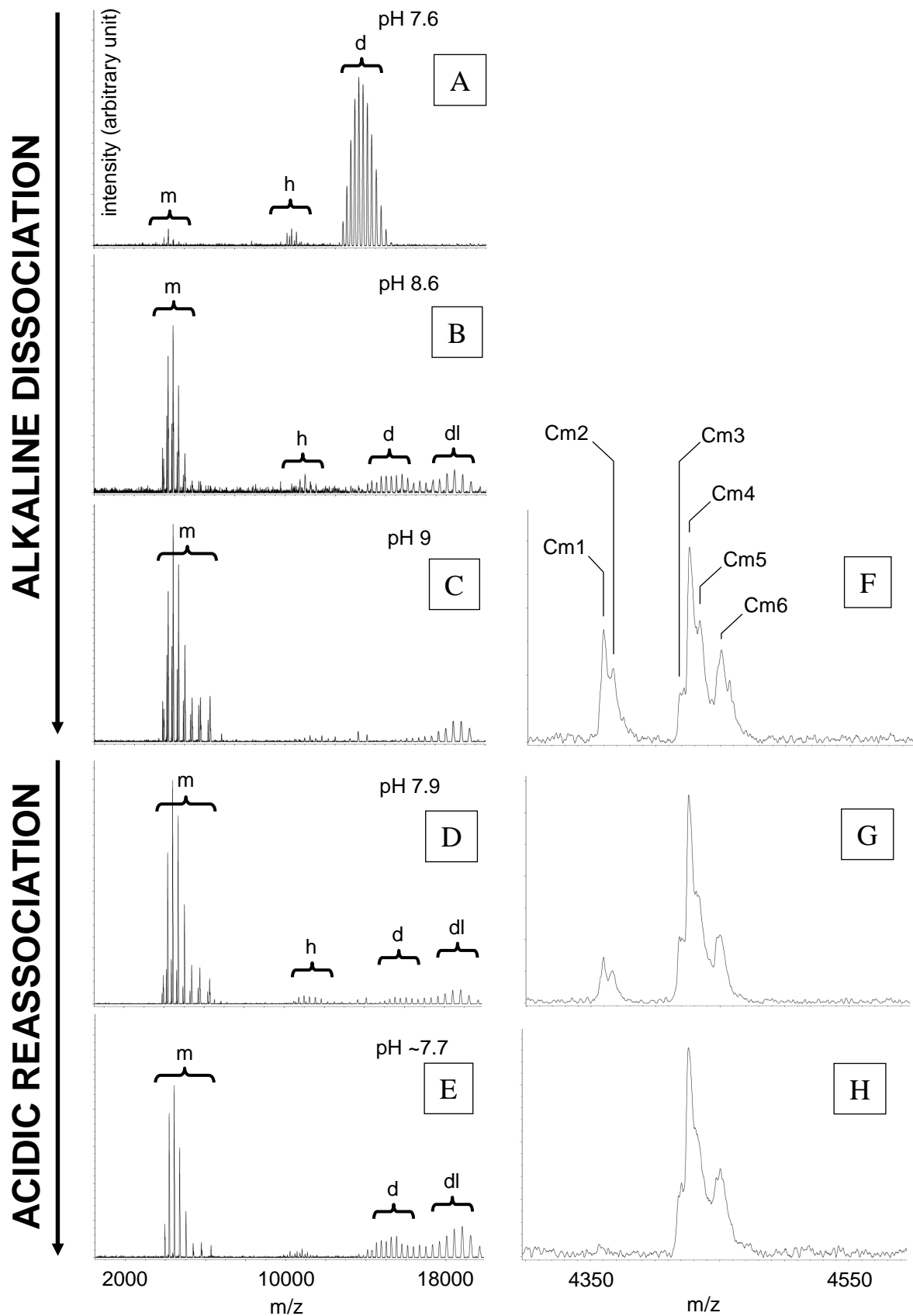


Figure 2: Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin by TEA and formic acid

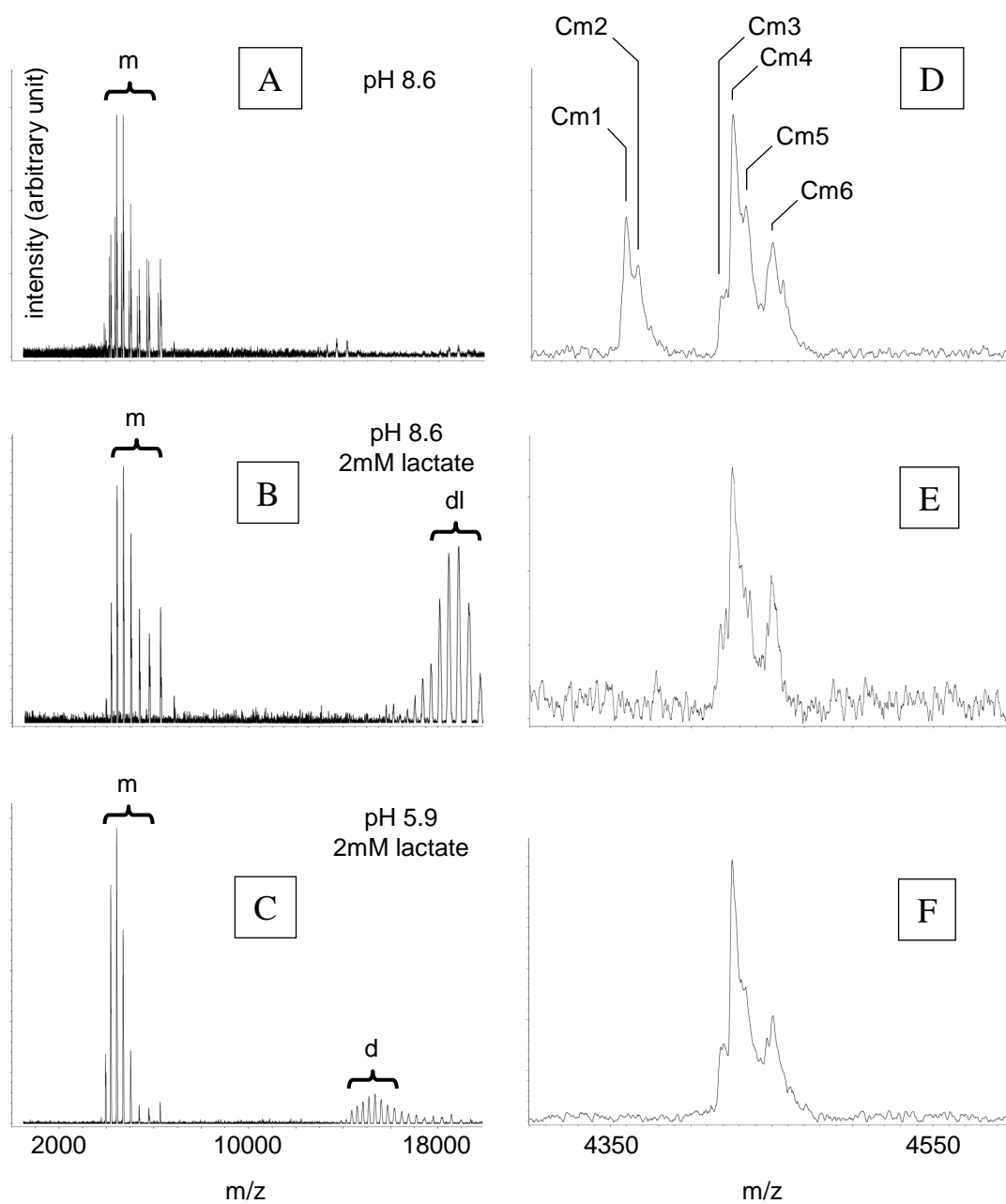


Figure 3: Acidic reassociation of *Carcinus maenas* hemocyanin by L-lactic acid

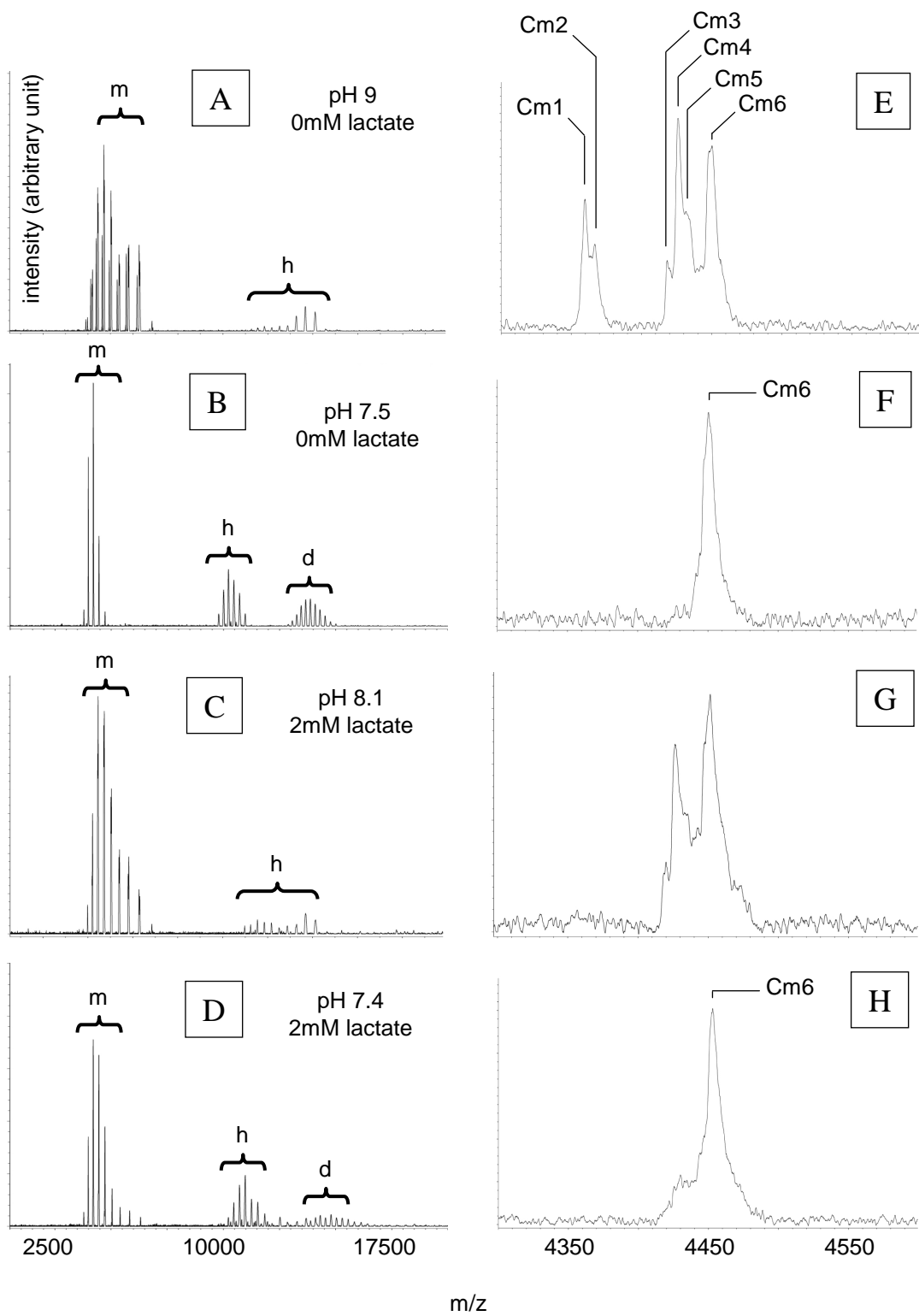


Figure 4: Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin coupled with chelation of divalent cations by EDTA

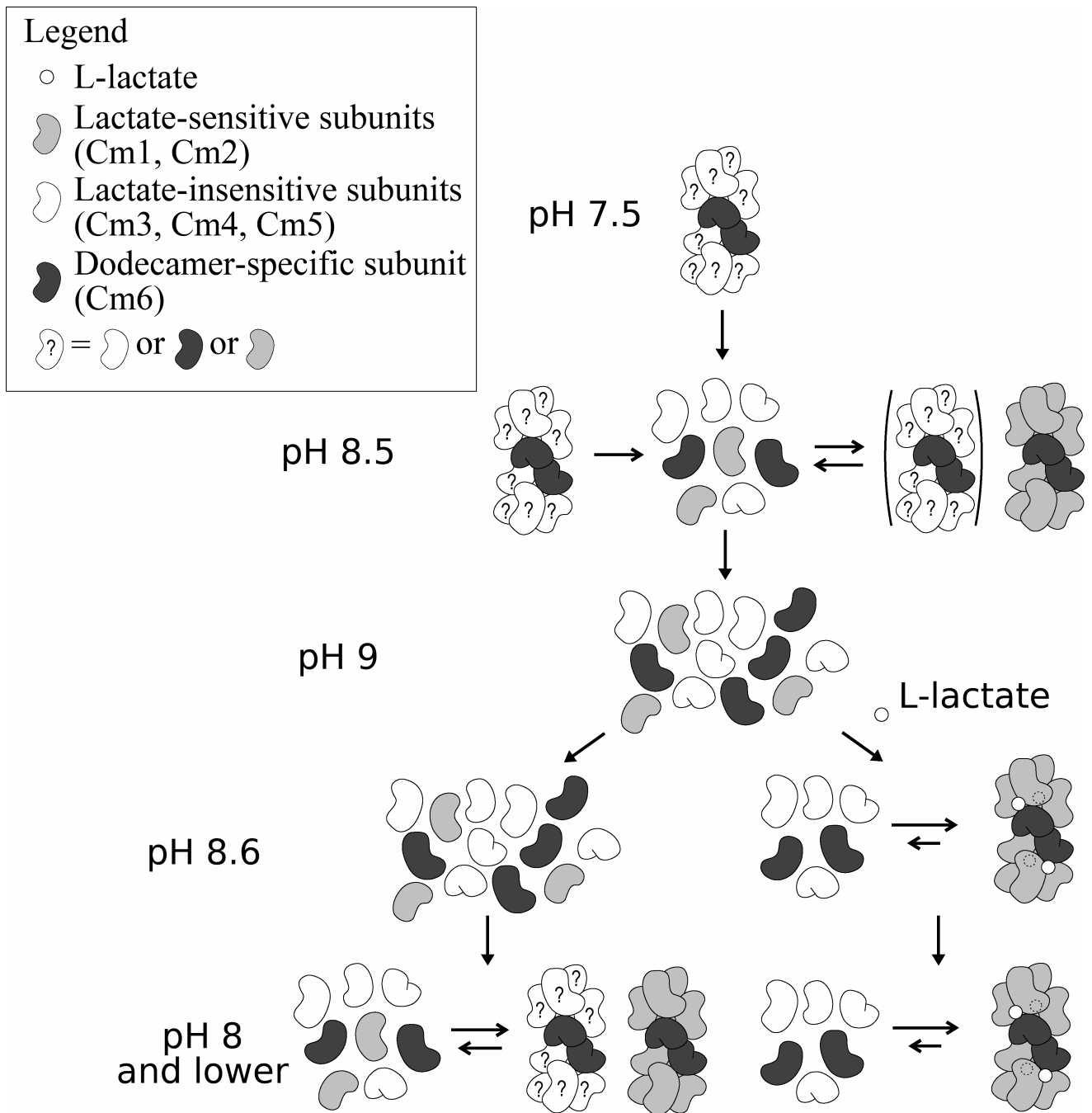


Figure 5: Summary of the dissociation and association steps observed under alkalization by TEA and acidification by formic acid or addition of L-lactate